The IgG Subclass Responses Induced by Wild-type, Cold-adapted and Purified Haemagglutinin from Influenza Virus A/Queensland/6/72 in CBA/CaH Mice

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SUMMARY

The IgG subclass and IgA responses were investigated in CBA/CaH mice after inoculation with wild-type (wt) and cold-adapted (ca) derivatives of influenza A/Queensland/6/72 virus, and with purified haemagglutinin (H3) derived from the wt strain of the same virus. Intranasal inoculation of the wt and ca viruses resulted in responses dominated by IgG2a in serum, saliva and lung secretions, whereas an intramuscular injection of purified H3 elicited the production of all four IgG subclasses in serum and IgG2b and IgG3 in saliva and lung secretions. The source of IgG on mucosal surfaces was from local production and was not a transudate from serum, as demonstrated by the lack of albumin in saliva and lung secretions, and by the appearance in saliva and lung samples of IgG subclasses not present in serum at the time of sampling. The level of IgA on mucosal surfaces was influenced by the growth restrictions of intranasally inoculated ca virus, resulting in higher levels of IgA in saliva, whereas wt virus, able to replicate at higher temperatures, induced higher levels of IgA in lung secretions. The purified H3 inoculated by the intramuscular route elicited lower levels of IgA in serum, saliva and lung secretions than either the wt or ca viruses.

INTRODUCTION

Immune responses to influenza infection have been studied extensively in the murine system. It has been demonstrated that (i) the production of IgG antibodies to the strain-specific determinants on the haemagglutinin (HA) induces significant host protection (Virelizier et al., 1979; Virelizier, 1975), (ii) cell-mediated immune responses exert a significant effect in limiting virus growth and persistence (Tannock & Paul, 1987; Ada & Jones, 1986), (iii) non-specific components of the immune system such as macrophages, natural killer (NK) cells, interferon and complement are important in limiting virus spread and initiating recovery (Mak et al., 1983; Yap et al., 1979; Rodgers & Mims, 1981, 1982; Hicks et al., 1978) and (iv) the use of inactivated virus preparations does not induce heterotypic immunity, which is normally seen after infection with live virulent or attenuated virus strains (Liew et al., 1984).

Previous studies in man have shown that inactivated virus preparations offer less protection than live attenuated virus vaccines (Freestone et al., 1972; Mackenzie et al., 1975). However the reasons for this have not been fully determined (Mackenzie, 1977). Infectious virus possibly induces cross-reactive cytotoxic T cells or distinct antibody isotypes in the serum and respiratory tracts of infected animals (Webster & Askonas, 1980). Neutralization of virus is affected by antibody isotypes, and Taylor & Dimmock (1985a, b) found large differences between monomeric (IgG and IgA) and oligomeric (IgM and secretory IgA) antibody classes. Lemercier et al. (1977) and Liew et al. (1984) showed that there is a difference between the IgG subclasses.
induced by influenza virus in mice, but more extensive studies are required to investigate fully the IgG subclass responses induced by different virus preparations.

In murine serum there are four IgG subclasses which could participate in virus neutralization. They vary considerably in their physical and biochemical properties (Burton, 1985; Spielberg, 1974; Fahey et al., 1964). For example, murine IgG1 activates complement less efficiently than do IgG2a and IgG2b (Spielberg, 1974); IgG2a binds with a greater avidity than the other subclasses to Fc receptors on mouse macrophages (Burton, 1985); IgG2b has the greatest segmental flexibility about its hinge region in comparison to the other three subclasses, which possibly restricts the functions of its Fc region (Burton, 1985); IgG3 does not activate complement (Winklehake, 1978). The IgG subclass induced in response to any antigen, either in serum or through secretion onto mucosal surfaces, would therefore dictate the immune mechanisms by which antibody neutralizes or clears antigen from the host. Indeed, Schulman (1975) has reported that the levels of IgG on mucosal surfaces in the lower respiratory tract after intranasal inoculation of infectious influenza virus may indicate that it is the most important antibody subtype involved in protection in the lung.

The purpose of this study, therefore, was to determine the IgG subclass responses in mice following infection with virulent [wild-type (wt)] and attenuated [cold-adapted (ca)] influenza A/Queensland/6/72 (H3N2) virus which was inoculated intranasally (i.n.), and to purified haemagglutinin (H3) derived from the wt of the same strain of virus, which was administered intramuscularly (i.m.). IgG subclass and IgA responses were investigated in the serum, saliva and lung secretions of inoculated mice and results are discussed in terms of the known biochemical and physiological properties of the IgG subclasses.

METHODS

Viruses. Viruses used in this study were A/Queensland/6/72 (H3N2), designated wt, and a cold-adapted derivative, designated ca (both were kindly supplied by Dr G. Tannock, Faculty of Medicine, University of Newcastle, New South Wales, Australia). Virus stocks were grown in 10-day-old embryonated chicken eggs and incubated at 37 °C for the wt virus and 33 to 34 °C for the ca virus. After 48 h the infected allantoic fluid was harvested, precipitated using 8% (w/v) polyethylene glycol 6000, as described by Cockrell & Monstratos (1982), and purified on 40 to 60% (w/v) discontinuous sucrose gradients. The visible bands of virus were removed and dialysed overnight against 0·1 m-phosphate buffer pH 7·4 containing 0·15 m-NaCl (PBS). The resulting stocks were divided into samples and stored at −70 °C.

IgG subclass determination. An ELISA was developed to analyse samples for their IgG subclass content using microtitration plates coated with purified H3 from wt virus. After dilution to 20000 haemagglutination (HA) units/ml (equivalent to 15 μg protein/ml) in carbonate buffer pH 9·6, 100 μl of the H3 solution was added to 96-well plates (Flow Laboratories) and incubated overnight at 4 °C. Samples to be analysed were diluted 1:200 in PBS containing 0·01% (v/v) Tween and 1% (w/v) bovine serum albumin (PBST), and added to the 96-well plates after they had been washed three times with PBST. After a further overnight incubation at 4 °C, the plates were washed again and rat monoclonal anti-mouse IgG subclass antisera (Cooper Biomedical, Pa., U.S.A.) were added and incubated at 4 °C for 6 h. The plates were washed three times with PBST and incubated overnight at 4 °C with alkaline phosphatase-conjugated goat anti-rat IgG (Bio-Rad) previously diluted in a 1:10 dilution of normal mouse serum. The plates were washed with PBST and a 1 mg/ml solution of p-nitrophenol phosphate was added. The absorbance at 405 nm was then determined using a Multiskan plate reader (Flow Laboratories). The anti-HA response was expressed in ELISA units after referring to a standard curve constructed using a standard positive serum and a four-parameter logistic model (Rodbard & Hutt, 1974). The ELISA values for normal mouse serum controls were deducted from all sample readings.

Specificity of anti-mouse IgG subclass monoclonal antisera. Specific mouse IgG subclasses were either isolated from mouse hybridomas CIRU 215 (IgG1), OKT3 (IgG2a) and OKM1 (IgG2b), all kindly donated by Associate Professor K. Turner, Princess Margaret Hospital for Children, Subiaco, Western Australia, or purchased from Miles Laboratories. Ascitic fluid from the aforementioned hybridomas was purified on Protein A-Sepharose 4B (Pharmacia) by the method of Ey et al. (1978). The specificities of the anti-mouse monoclonal antisera were then determined using the isolated mouse subclasses in an ELISA. Subclasses were coated onto plates at a concentration of 10 μg/ml and the ELISA was performed, as outlined above, using a goat anti-rat IgG–alkaline phosphatase conjugate diluted in normal mouse serum.

No significant cross-reactivity was found between the monoclonal IgG subclass antisera. When tested against the different subclasses bound to test plates, absorbance values of 0·2 to 0·3 were detected; these were within background levels.
IgA levels. The level of IgA in samples was determined by ELISA using rabbit anti-mouse IgA, Fc-specific (Nordic Immunological Laboratories, Maidenhead, U.K.) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Cooper Biomedical) on purified H3-coated 96-well plates, using the same procedure as that employed for the IgG subclass ELISA.

Albumin content. The albumin content of lung lavages and pilocarpine-induced salivary secretions was assessed using plates coated with rabbit anti-mouse albumin (Nordic Immunological Laboratories) in an ELISA capture assay. The bound albumin was detected using a rabbit anti-mouse albumin–alkaline phosphatase conjugate (prepared by the method of Voller et al., 1976). Purified mouse albumin (Nordic Immunological Laboratories) was used as a positive control.

Purification of H3. H3 from wt virus was prepared by incubating virus at room temperature with 1 to 1.5% (v/v) Nonidet P40 (NP40) for 60 to 70 min. After centrifuging to clarify the solution, the resulting supernatant was layered onto continuous 5 to 20% (w/v) sucrose gradients containing 0.1% (v/v) NP40. The gradients were centrifuged for 7-5 h at 260,000 g and then separated into 13 fractions. The fractions shown to contain HA activity were further purified on an oxamic acid–agarose (Sigma) affinity column to remove any contaminating neuraminidase (Phelan et al., 1980). NP40 was then removed with Bio-Beads SM-2 (Bio-Rad) by the method of Holloway (1973) and the resultant sample was dialysed against PBS. The H3 was then analysed for purity by HA, neuraminidase assay and SDS–PAGE.

SDS–PAGE. Samples were analysed on 10% polyacrylamide slab gels (Fig. 1), according to the method of O’Farrell (1974). The Mₙ standards (Pharmacia) used were carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200) and phosphorylase B (92,500). Gels were stained for protein using the method of Burk et al. (1983).

Neuraminidase assay. The neuraminidase activity of samples was determined using N-acetylneuraminic-lactose (Sigma) as substrate according to the method of Cuatrecasas & Illiano (1971).

Mouse inoculations. Ten-week-old CBA/CaH mice, obtained from the Animal Resource Centre of Western Australia, were used throughout these studies. At the time of inoculation every mouse was anaesthetized by an intraperitoneal injection of 0.15 to 0.20 ml of pentobarbitone sodium (Ceva Chemicals, Australia), in PBS. Three different virus inocula were used as follows: the wt virus and the ca virus were inoculated i.n. and the purified H3 i.m. A dose of 100 HA units (0.3 μg protein) of wt and ca viruses and 100 HA units (0.075 μg protein) of purified H3 were administered to individual mice. Virus inocula were instilled i.m. in a total volume of 0.05 ml using a calibrated 0.025 ml dropper pipette. Inoculations performed i.m. were delivered using a 1 ml syringe in a total volume of 0.1 ml. Control mice received the appropriate volumes of PBS. All mice were reinoculated 8 weeks after the primary injection of virus.

Preparation of lung, nasal and serum samples. Nasal and salivary secretions were stimulated by injecting anaesthetized mice i.m. with 0.1 ml of a 2% (w/v) solution of pilocarpine hydrochloride in 0.5% (v/v) hypromellose (Holt et al., 1987). The resulting secretions were collected over a 5 to 7 min period. The mice were then bled, killed and their lungs were lavaged with 1 ml of PBS. At each time point samples from five mice were pooled and stored at −20 °C before analysis. All experiments were repeated three times and the average of the readings was taken.

RESULTS

Virus HA-specific IgG subclass responses

Serum

The serum IgG subtype responses of mice to i.n. infection initiated with wt and ca viruses, and to i.m. injection with purified viral HA, are shown in Fig. 2. Infection of mice with wt virus resulted in a response of predominantly the IgG2a subtype (at least five- to 10-fold higher than any other subclass), although varying levels of IgG1, IgG2b and IgG3 were also detected (Fig. 2a). After exposure to a second dose of virus, the IgG2a response remained high, but of the other subtypes only IgG1 was significantly elevated although at a level 10-fold less than IgG2a; there was no IgG2b and little IgG3. Infection with a primary dose of ca virus also resulted in a response which was principally IgG2a (Fig. 2b) but, although the level of this subtype remained high after a second exposure to ca virus (10-fold higher than any other subclass), the response of the other subtypes differed markedly from those observed with wt virus, being primarily IgG2b and IgG3 rather than IgG1. When purified HA was the immunogen, there was a lower response after primary injection than with live virus infections (Fig. 2c) and no subtype predominated. The response consisted of IgG1, IgG2a and IgG2b 1 week post-immunization, IgG2a, IgG2b and IgG3 after 3 weeks and only a very low level of IgG3 after 7 weeks. Thus both the level and
duration of the response were less than found after exposure to live virus, and the subtype balance was different. A second injection of purified HA induced significant levels of all four subtypes which persisted for the following 10 weeks.

**Lung**

The IgG subtype responses in lung lavage fluids are depicted in Fig. 3. The responses observed after primary and secondary infection with wt or ca viruses were dominated by IgG2a (at least 10-fold higher than the other subclasses for both viruses), the levels being slightly higher with wt. The wt virus also elicited the other three subclasses but, although the IgG1 subclass was
constantly present, the appearance of IgG2b and IgG3 subclasses appeared to alternate following re-exposure to the virus (Fig. 3a). The response following infection with ca virus was delayed, with IgG2a not evident until 3 weeks and IgG2b and IgG3 until 7 weeks after infection (Fig. 3b). The IgG2b and IgG3 subclasses were also observed after re-exposure to ca virus but, in contrast to the results with wt virus, there was no evidence of 'switching' between these two subclasses. Purified H3 induced lower levels of IgG in the lung lavage fluids than either wt or ca viruses (Fig. 3c), and the response following primary injection was totally confined to the IgG2b and IgG3 subtypes. A second immunization with purified H3 induced subtypes IgG2a, IgG2b and IgG3, with IgG3 predominating. Four weeks later, however, only IgG2b was detected. Twelve weeks after the second immunization, IgG1 was observed for the first time, with IgG2a and IgG2b.

**Nasal and salivary secretions**

The IgG subtype responses in nasal and salivary secretions are shown in Fig. 4. Inoculation of mice with wt virus elicited an immune response dominated by IgG2a, with varying levels of IgG2b and IgG3 at 3 and 7 weeks post-inoculation. After a second dose of virus, IgG2a still predominated, with IgG1 making its first appearance at 12 weeks after the second infection. The response to ca virus was delayed, with only IgG2a being detected at 3 weeks post-primary inoculation and switching to IgG2b and IgG3 by 7 weeks. After a second dose of virus, IgG2a predominated, with varying levels of the other three subclasses, but 12 weeks after the second inoculation only IgG2a and IgG2b remained. In contrast, the purified HA induced only IgG2b, even after a second inoculation.
FIG. 4. Saliva IgG responses to wt (a) and ca (b) H3N2 influenza viruses and purified H3 (c). Symbols and time of reinoculation as in Fig. 2.

FIG. 5. IgA responses to wt (a) and ca (b) H3N2 influenza viruses and purified H3 (c) in lung lavage (II), saliva (●) and serum (■) samples. Mice were reinoculated (↓) with virus 8 weeks after receiving a primary inoculation.

**Albumin levels**

Albumin levels in lung lavage fluids were 0.1 to 0.025% of those found in serum (results not shown), indicating that the contamination of lung lavage samples with serum albumin was minimal. It was assumed therefore that damage to the lung during sampling was also minimal and that contamination of lavage samples with serum antibodies was negligible.

**Virus-specific IgA responses**

Of the three virus preparations examined, the greatest IgA responses in the respiratory tract were obtained with the wt virus preparation (Fig. 5a to c). The purified H3 was the least effective in stimulating IgA production, as judged by the analysis of saliva and lung lavage fluids after two inoculations of virus preparation, although serum levels approached those obtained using the wt and ca viruses. This would seem to be a reflection of the route of inoculation i.e. inoculation i.m. is not the most effective way of stimulating IgA secretions onto mucosal surfaces (McDermott et al., 1982).

The wt virus stimulated higher levels of IgA in lung lavage fluids than did the ca virus but in saliva this situation was reversed. Two doses of wt virus were required to stimulate IgA levels similar to those induced by ca virus in saliva. These levels of IgA would appear to reflect the ability of the two viruses to replicate in different environments, i.e. the upper respiratory tract which is at a slightly lower temperature than the lung favours the replication of ca virus, whilst wt virus proliferates at the elevated temperatures in the lung (Tannock et al., 1984; Nai-Ki et al., 1982).
DISCUSSION

Differences were detected in the IgG subclass responses in the serum, saliva and lung secretions of mice after inoculation with wt influenza A/Queensland/6/72 virus, a ca derivative of this virus and purified H3 from the same strain. The wt and ca viruses inoculated i.n. initiated responses in serum and lung dominated by IgG2a. The wt virus also induced a prolonged IgG1 response in comparison to the ca virus response in which IgG1 was present only after secondary inoculation, and then only transiently, with only IgG2b and IgG3 present in the later stages of the response. The predominance of IgG2a in serum responses to i.n. inoculation of wt and ca viruses is in agreement with the results of Coutelier et al. (1987), who used a variety of DNA and RNA viruses. In contrast, no predominance of any particular subclass was observed in the serum of mice inoculated i.m. with purified H3; all four subtypes were observed at high levels after the secondary inoculation, whereas in the lungs and saliva of these mice IgG2b was present throughout the experimental period. IgG3 was not detected from 14 weeks after primary inoculation.

The appearance of IgG on mucosal surfaces initiated by the wt and ca viruses appears to result from the local production of antibody as demonstrated by the lack of some subclasses in serum and their appearance in lung lavage and saliva samples. The lack of serum albumin in samples indicated that contamination with serum antibodies at the time of sampling was negligible. Evidence as to the source of IgG on mucosal surfaces is conflicting. Local production of antibody in mice has been demonstrated by Owens et al. (1981) and Jones & Ada (1986). However, other studies in humans (Wagner et al., 1987; Clements & Murphy, 1986) have suggested that IgG on mucosal surfaces is the result of a transudate from serum. The ability of the four murine IgG subclasses to cross mucosal membranes from the serum is not established, but the selective appearance of a particular subclass on mucosal membranes could influence the mechanisms by which virus is neutralized and the efficiency of these mechanisms.

The responses in lung and saliva detected for the wt and ca viruses in mice inoculated i.n. were dominated by IgG2a. In contrast, mice which had been inoculated i.m. with purified H3 produced a response that elicited IgG2b over a longer period of time with a smaller proportion of IgG2a. The prevalence of IgG2a in the responses to the wt and ca viruses and IgG2b in the response to purified H3 is indicative of the importance of the IgG2a and IgG2b subclasses to the antibody-mediated immune mechanisms for the elimination of virus from the host.

The role of IgG2a and IgG2b in the clearance of influenza virus was demonstrated by Reale et al. (1985) using X-linked defective mice. These mice show decreased levels of IgG1 and IgG3 in both primary and secondary responses to influenza virus. Despite these defects no difference was observed in terms of LD_{50}, lung lesions or lung virus titres from non-defective strains after aerosol infection. The properties of IgG2a and IgG2b which make them important in the elimination of influenza virus can be stated as follows. (i) IgG2a and IgG2b activate complement efficiently, whereas IgG1 and IgG3 are unable to do so (Burton, 1985; Winkelhake, 1978). Increased pulmonary pathology and delayed viral clearance in the lungs of decomplemented mice, in comparison with controls infected with influenza virus, was observed by Hicks et al. (1978). Indeed, using mouse monoclonal anti-dansyl antibodies Oi et al. (1984) were able to demonstrate that IgG1 required 10-fold more antigen to trigger complement activation than did IgG2a and IgG2b. (ii) The selective enhancement of IgG2a Fc receptors by the activation of macrophages has been shown to depress the expression of Fc receptors for the other three subclasses (Ezekowitz et al., 1983). In serum and lung, activated macrophages have been shown to contribute towards protection against influenza infection (Fujisawa et al., 1987; Tsuru et al., 1987). (iii) Granular lymphocytes have been shown to carry out cytotoxic activities through Fc receptors for IgG1, IgG2a and IgG2b (Lopez et al., 1983). The generation of NK cells which are important in the recovery from and protection against influenza infection (Hashimoto et al., 1983; Ada & Jones, 1986) have been shown in mice to correlate with large granular lymphocytes (Natuk & Welsh, 1987) and the cytotoxic activities of eosinophils and neutrophils (granular lymphocytes).

The similarity of the IgG subclass profiles observed for the wt and ca viruses and their difference from the purified H3 preparation is possibly explained by the differences in the
immunizing antigens. Slack et al. (1980) postulated that the intrinsic properties of an antigen, unrelated to antigenic determinants binding to cell receptors, cause selective stimulation of B cells at a particular stage of differentiation to produce a given class of immunoglobulin. The presentation of H3 antigen to B cells could differ when the haemagglutinin is inoculated as a purified protein, in contrast to its inoculation as part of an infectious virus. In addition, the growth restrictions of ca viruses in comparison to wt viruses (Tannock & Paul, 1987) could also influence the production of IgG. The ca virus due to its temperature restrictions is not able to attain the same titres in lung and serum as the wt virus. Higher titres of wt virus in the lungs of infected mice as shown by Nai-Ki et al. (1982) could promote B cells to produce IgG1 in preference to IgG2b and IgG3, which are induced in response to ca virus. However, the dominance by IgG2a in the responses to both the wt and ca viruses supports the proposal by Maasab (1967) for the use of ca viruses as vaccine strains.

In conclusion, it has been demonstrated that different influenza virus preparations produce different IgG subclass profiles in mice. Those induced are observed in the body fluids tested, i.e. serum, lung and saliva. Further studies in other strains of mice are therefore required in order to confirm the association between IgG subclass responses and those found in this study, and work on the neutralizing abilities of the individual subclasses also needs to be undertaken.

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IgG subclass responses to influenza A virus

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